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Overexpression of Regucalcin Suppresses Cell Proliferation in Cloned Rat Hepatoma H4-II-E Cells: Involvement of Intracellular Signaling Factors and Cell Cycle-Related Genes

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Abstract The role of regucalcin, which is a regulatory protein in intracellular signaling pathway, in the regulation of cell proliferation was investigated by using the cloned rat hepatoma H4-II-E cells overexpressing regucalcin. The hepatoma cells (wild type) and stable regucalcin/pCXN2 transfectants were cultured for 72 h in a medium containing 10% fetal bovine serum (FBS) to obtain subconfluent monolayers. The proliferation of cells was significantly suppressed in transfectants cultured for 24–72 h. The proliferation of wild-type cells was significantly inhibited when the cells were cultured for 72 h in a medium containing an inhibitor of transcriptional activity or protein synthesis. Such an effect was not seen in transfectants. The presence of various inhibitors of protein kinase including PD 98059 (10^{-7} or 10^{-6} M), dibucaine (10^{-6} M), wortmannin (10^{-8} or 10^{-6} M), or genistein (10^{-5} M) caused a significant inhibition of the proliferation of wild-type cells. These inhibitory effects were not seen in transfectants. Staurosporine ($10^{-8} - 10^{-7}$ M) significantly inhibited the proliferation of wild-type cells and transfectants. Also, the effect of vanadate (10^{-5} M), an inhibitor of protein tyrosine phosphatase, or Bay K 8644 (10^{-6} M), an agonist of calcium entry into cells, in inhibiting the proliferation of wild-type cells was not observed in transfectants. Moreover, the proliferation of wild-type cells was significantly inhibited in the presence of roscovitine (10^{-7} or 10^{-6} M) or sulforaphane (10^{-7} M), which induces cell-cycle arrest. Such effect was not seen in transfectants. The inhibitory effect of sodium butyrate (8.3×10^{-4} M) on proliferation of wild-type cells was also induced in transfectants. Gene expression in hepatoma cells cultured for 72 h with 10% FBS was determined by using reverse transcription-polymerase chain reaction (RT-PCR). The expression of p21 mRNA was significantly enhanced in transfectants, while cdc2a and chk2 mRNA expression were not significantly changed. Insulin-like growth factor-I (IGF-I) mRNA expression was significantly suppressed in transfectants. This study demonstrates that overexpression of regucalcin has a suppressive effect on cell proliferation that is partly mediated through various intracellular signaling-related factors, and that the effect may be partly involved in the change in p21 or IGF-I mRNA expression. The finding further supports that regucalcin plays an important role as a suppressor in the enhancement of cell proliferation. J. Cell. Biochem. 95: 1169–1177, 2005. © 2005 Wiley-Liss, Inc.

Key words: regucalcin; cell proliferation; p21; IGF-I; cell signaling; hepatoma cells

Regucalcin, which was found as a novel Ca^{2+}-binding protein not including the EF-hand motif [Yamaguchi and Yamamoto, 1978; Yamaguchi, 1992 in reviews; Shimokawa and Yamaguchi, 1993], has been demonstrated to play a multifunctional role as an inhibitory protein an intracellular signaling process in cells [Yamaguchi, 2000a,b, 2005; reviews] in recent years. The gene of regucalcin is highly conserved in vertebrate species [Shimokawa and Yamaguchi, 1993; Misawa and Yamaguchi, 2000]. The rat and human regucalcin genes are localized on chromosome X [Shimokawa et al., 1995; Thiselton et al., 2002]. Regucalcin messenger ribonucleic acid (mRNA) and its protein are greatly present in liver and kidney cortex [Shimokawa and Yamaguchi, 1992; Yamaguchi and Isogai, 1993]. The expression of regucalcin mRNA is mediated through Ca^{2+}-signaling mechanism [Murata and Yamaguchi, 1999;

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Yamaguchi and Nakajima, 1999]. AP 1 and NFI-A1 have been found to be transcriptional factors for the enhancement of regucalcin gene promoter activity [Murata and Yamaguchi, 1999; Misawa and Yamaguchi, 2002].

Regucalcin plays a role in the maintenance of intracellular Ca^{2+} homeostasis, the inhibitory regulation of various Ca^{2+}-dependent protein kinase, tryrosine kinases, protein phosphatases, nitric oxide (NO) synthase, protein synthesis, nuclear DNA and RNA synthesis in many cell types [Yamaguchi, 2000a,b, 2005]. Regucalcin transgenic rats have been shown to induce bone loss [Yamaguchi et al., 2002] and hyperlipidemia [Yamaguchi et al., 2004], suggesting its pathophysiologic role. Regucalcin may play a pivotal role in the regulation of cell function in body.

Regucalcin has been shown to translocate to the nucleus of rat liver [Tsurusaki et al., 2000; Laz et al., 2004], and it has been demonstrated to regulate nuclear function in regenerating rat liver with proliferative cells [Tsurusaki and Yamaguchi, 2002a,b; Tsurusaki and Yamaguchi, 2003a], suggesting that the protein has a suppressive effect on the overexpression of proliferation of liver cells. Recent study has demonstrated that endogenous regucalcin has been shown to suppress the enhancement of protein kinase [Inagaki and Yamaguchi, 2001a] and protein phosphatase activities [Inagaki et al., 2000] in the cloned rat hepatoma H4-II-E cells with proliferation, supporting a role of regucalcin in the regulation of cell proliferation [Inagaki and Yamaguchi, 2001b].

Overexpression of regucalcin, moreover, has been shown to have a suppressive effect on DNA synthesis and cell proliferation in the cloned rat hepatoma H4-II-E cells [Misawa et al., 2002]. Overexpression of regucalcin modulates the expression of tumor-related genes (*c-myc*, Ha-*ras*, or *p53*) in the cloned rat hepatoma H4-II-E cells [Tsurusaki and Yamaguchi, 2003b]. Regucalcin may modulates the transcriptional process by binding to protein and DNA in the liver nucleus [Tsurusaki and Yamaguchi, 2004]. The role of regucalcin in the regulation of cell proliferation has not been fully clarified, however.

The present study was undertaken to determine the involvement of intracellular signaling factors in the regulation of cell proliferation in the cloned rat hepatoma H4-II-E cells overexpressing regucalcin. We found that overexpression of regucalcin has a suppressive effect on cell proliferation that is mediated through various intracellular signaling-related factors, and that the effect is partly involved in the change in p21, which participates in cell cycle arrest, and IGF-I mRNA expression. The finding further supports that regucalcin plays an important role as a suppressor in the enhancement of cell proliferation.

MATERIALS AND METHODS

Chemicals

α-Minimum essential medium (α-MEM) and penicillin–streptomycin solution (5,000 U/ml penicillin; penicillin; 5,000 μg/ml streptomycin) were obtained from Gibco Laboratories (Grand Island, NY). Fetal bovine serum (FBS), 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), cycloheximide, PD98059, dibucaine, staurosporine, wortmannin, genistein, Bay K 8644, sodium butyrate, roscovitine, and sulforaphane were obtained from Sigma Chemical Co. (St. Louis, MO). Calcium chloride, vanadate, and other chemicals were purchased from Wako Pure Chemical Co. Ltd. (Osaka, Japan). Reagents used were dissolved in distilled water or ethanol, and some reagents were passed through ion-exchange resin to remove metal ions.

Regucalcin Transfectants

Regucalcin transfectants, which are overexpressing regucalcin in the cloned rat hepatoma H4-II-E cells, were used in this experiments as reported previously [Misawa et al., 2002]. The cDNA encoding rat regucalcin was isolated and cloned into the pBluscript vector [Shimokawa and Yamaguchi, 1993]. The regucalcin cDNA contains *P*st I site downstream of the translational stop codon, and a *P*st I site and an *Eco*RI upstream of the regucalcin cDNA. The *Eco*RI fragment (containing the complete coding cDNA) was cloned into the *Eco*RI site of the pCXN2 expression vector [Niwa et al., 1991]. The resultant plasmid was designated as regucalcin/pCXN2 [Misawa et al., 2002].

For transient transfection assay, the H4-II-E cells were grown on 35-mm dishes to approximately 70% confluence. Each of regucalcin/pCXN2 and pCXN2 vector alone was transfected into H4-II-E cells using the synthetic cationic lipid components, a Tfx-20 reagent, according to the manufacturer's instructions (Promega, Madison WI). After 24 h, neomycin

(1.0 mg/ml Geneticin G418, Sigma) was added to cultures for selection and cells were plated at limiting dilution. Multiple surviving cloned were isolated, transferred to 35-mm dishes, and grown in the medium without neomycin. Regucalcin was stably expressed in the transfectants [Misawa et al., 2002]. In experiments, transfectants were cultured for 72 h in α-MEM containing 10% FBS.

Cell Culture

The cloned rat hepatoma H4-II-E cells and the transfectant of H4-II-E cells (1.0×10^5) were maintained for 72 h in α-MEM supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin in humidified 5% CO_2/95% air at 37°C to obtain subconfluent monolayers [Misawa et al., 2002]. In experiments, cells were cultured for 72 h in a medium containing either vehicle or various factors in the presence of 10% FBS. After culture, cells were washed three times with phosphate-buffered saline (PBS), and the number of cells was counted.

Cell Counting

After trypsinization of each of the culture dishes using 0.2% trypsin plus 0.02% EDTA in Ca^{2+}/Mg^{2+}-free PBS for 2 min (min) at 37°C, cells were collected and centrifuged in a PBS at 100*g* for 5 min. The cells were resuspended on PBS solution and stained with eosin. Cell numbers were counted under a microscope using a Hemacytometer plate. For each dish, we took the average of two countings.

Determination of Specific mRNA by RT-PCR

Total RNAs were prepared as described previously [Chomczyshi and Sacchi, 1987]. After culture, cells were washed three times with ice-cold PBS, and then cells were homogenized in buffer solution containing 4 M guanidinium thiocyanate, 24 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and isoamyl alcohol, and the phases were separated by centrifugation at 10,000*g* for 20 min at 4°C. RNA located in the aqueous phase was precipitated with isoprepanol at −20°C. RNA precipitates were pelleted by centrifugation, and the pellets were redissolved in diethylpyrocarbonate-treated water.

RT-PCR was preformed with a Titam™ One Tube RT-PCR kit (Roche Molecular Biochemicals) as recommended by the supplier Primers for amplification of rat cell division cycle 2 homolog A (cdc2a) cDNA were: 5′-AAGCGAGGAAGAAGGAGTGCCCAG-3′ (sense strand, positions 214–237 of cDNA sequence) and 5′-TCCAAACGCTCTGGCAAGGCC-3′ (antisense strand, positions 542–562) [Shimizu et al., 1995]. The pair of oligonucleotide primers was designed to amplify a 349 bp sequence from the mRNA of rat cdc2a. Primers for amplification of rat checkpoint kinase 2 (chk2) cDNA were: 5′-CGGCTATGGGCTCTTCAGGATGG-3′ (sense strand, positions 367–409 of cDNA sequence) and 5′-CAAAGGCCATCTTTACCTCCCCACA-3′ (antisense strand, positions 795–819) [Chaturvedi et al., 1999]. The pair of oligonucleotide primers was designed to amplify a 453 bp sequence from the mRNA of rat chk2. Primers for amplication of rat p21 cDNA were: 5′-TCAGAGCCACAGGCACCATGTCC-3′ (sense strand, positions 81–103 of cDNA sequence) and 5′- CACTTCAGGGCTTTCTCTTGCAGAAGA- 3′ (antisense strand, positions 570–596) [Belinsky et al., 1996]. The pair of oligonucleotide primers was designed to amplify a 516 bp sequence from the mRNA of rat p21. Primers for amplication of rat IGF-I were: 5′-CGATGCCAGCCAGCTCCATCC-3′ (sense strand, positions 48–68 of cDNA sequence) and 5′-GGGTCGTTTACAGAGGAGGT-3′ (antisence strand, positions 731–750) [Casella et al., 1987]. The pair of oligonucleotide primers was designed to amplify a 703 bp sequence from the mRNA of IGF-I. For semiquantitative PCR, G3PDH was used as an internal control to evaluate total RNA input. Primers for amplification of glyceroaldehyde-3-phosphate dehydrogenase (G3PDH) cDNA were, 5′- GATTTGGCCGTATCGGACGC-3′ (sense strand) and 5′- CTCCTTGGAGGCCATGTAGG-3′ (antisense strand). The pair of oligonucleotide primers was designed to amplify a 977 bp sequence from the mRNA of rat G3PDH. RT-PCR was performed using reaction mixture (20 μl) containing 2 or 6 μg of total RNAs, supplied RT-TCR buffer, Titam™ enzyme mix (AMV and Expand™ High Fidelity), 0.2 mM dNTP, 5 mM dithiothreitol, 5 U RNase inhibitor, and 0.3 μM primers. Samples were incubated at 50°C for 30 min, and then amplified for 30 cycles under the following conditions: denaturation for 30 s at 94°C, annealing for 30 sec at 56°C, and extension for 60 s at 62.0–63.4°C. The amplified products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. Image density was quantified

with a FluoroImager SI (Amersham Biosciences, Piscataway, NJ).

Statistical Analysis

Data are expressed as the mean ± SEM. Statistical differences were analyzed using Student's t-test. P-values less than 0.05 were considered to indicate statistically significant differences. Also, we used an ANOVA multiple comparison test to compare the treatment groups.

RESULTS

Effect of Various Factors on Proliferation of Hepatoma Cells Overexpressing Regucalcin

The hepatoma cells (wild-type cells) or stable regucalcin/pCXN2 transfectants were cultured for 72 h in a medium containing 1.0 or 10% FBS to obtain subconfluent monolayers. The proliferation of the cells was significantly suppressed in transfectants cultured for 24, 48, or 72 h (Fig. 1). When hepatoma cells were cultured for 72 h in a medium containing DRB (10^{-6} M), an inhibitor of transcriptional activity, or cycloheximide (10^{-8} M), an inhibitor of protein synthesis, in the presence of 10% FBS, the proliferation of hepatoma cells (wild type) was significantly inhibited (Fig. 2). The inhibitory effect of DRB or cycloheximide was not seen in transfectant (Fig. 2).

The effect of various inhibitors of protein kinases on the proliferation of hepatoma H4-II-E cells was examined. Hepatoma cells were cultured for 72 h in a medium containing either vehicle, PD 98059 (10^{-7} or 10^{-6} M), an inhibitor of MAP kinase, dibucaine (10^{-7} or 10^{-6} M), an inhibitor of Ca^{2+}/calmodulin-dependent protein kinase, or staurosporine (10^{-8} or 10^{-7} M), an inhibitor of protein kinase C, in the presence of 10% FBS (Fig. 3). The presence of PD 98059 (10^{-7} or 10^{-6} M), dibucaine (10^{-6} M), or staurosporine (10^{-8} or 10^{-7} M) caused a significant

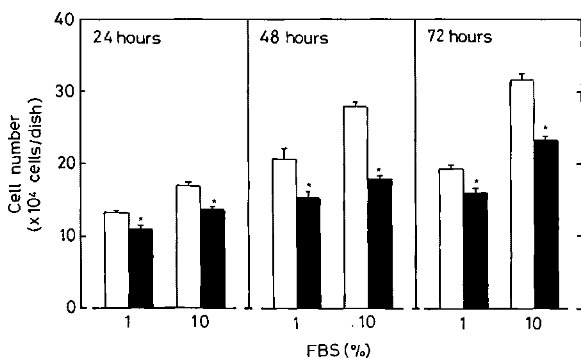

Fig. 1. Effect of FBS on the proliferation of cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells. Cells (1×10^5) were cultured for 24, 48, or 72 h in a medium containing either 1.0 or 10% FBS to obtain subconfluent monolayers. At each culture time, the number of cells was measured. Each value is the mean ± SEM of six experiments. *$P < 0.01$ compared with the value obtained from wild-type cells. White bars, wild-type cells; black bars, transfectants.

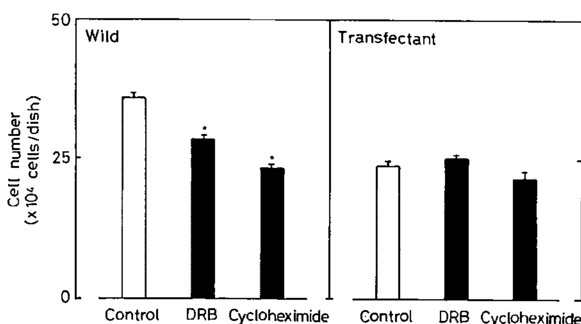

Fig. 2. Effect of DRB or cycloheximide on the proliferation of cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells. Cells (1×10^5) were cultured for 72 h in a medium containing either vehicle, DRB (10^{-6} M) or cycloeximide (10^{-8} M) in the presence of 10% FBS, and the number of cells was measured. Each value is the mean ± SEM of six experiments. *$P < 0.01$ compared with the control (none) value.

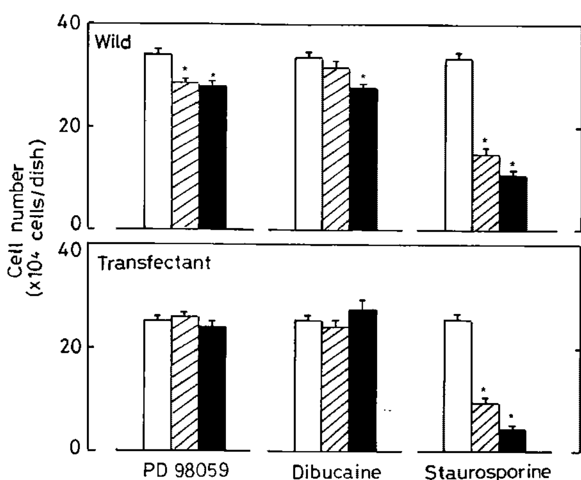

Fig. 3. Effect of PD 98059, dibucaine, or staurosporine on the proliferation of the cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells. Cells (1×10^5) were cultured for 72 h in a medium containing either vehicle, PD98059 (10^{-7} or 10^{-6} M), dibucaine (10^{-7} or 10^{-6} M) or staurosporine (10^{-8} or 10^{-7} M) in the presence of 10% FBS, and the number of cells was measured. Each value is the mean ± SEM of six experiments. *$P < 0.01$ compared with the control (none) value. Figures of PD 98059 represent white bars, control (none); hatched bars, 10^{-7} M; black bars, 10^{-6} M. Figures of dibucaine represent white bars, control (none); hatched bars, 10^{-7} M; black bars, 10^{-6} M. Figures of staurosporine represent white bars, control (none); hatched bars, 10^{-8} M; black bars, 10^{-7} M.

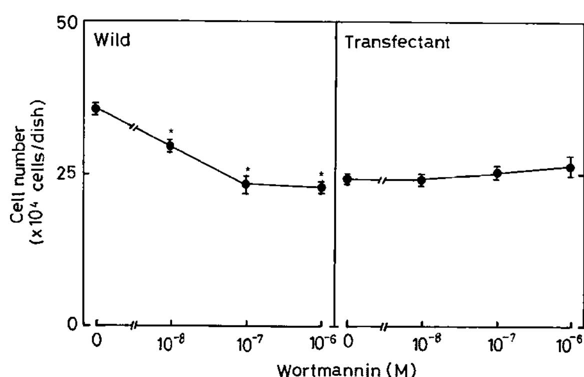

Fig. 4. Effect of wortmannin on the proliferation of cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells. Cells (1×10^5) were cultured for 72 h in a medium containing either vehicle or wortmannin ($10^{-8} - 10^{-6}$ M) in the presence of 10% FBS, and the number of cells was measured. Each value is the mean ± SEM of six experiments. $^*P < 0.01$ compared with the control (none) value.

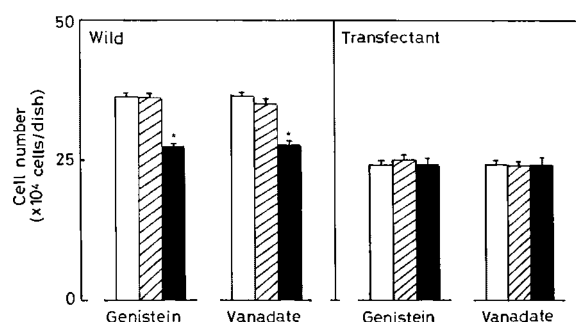

Fig. 5. Effect of genistein or vanadate on the proliferation of cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells. Cells (1×10^5) were cultured for 72 h in a medium containing either vehicle, genistein (10^{-6} or 10^{-5} M) or vanadate (10^{-6} or 10^{-5} M) in the presence of 10% FBS, and the number of cells was measured. Each value is the mean ± SEM of six experiments. $^*P < 0.01$ compared with the control (none) value. Figures of genistein represent white bars, control (none); hatched bars, 10^{-6} M; black bars, 10^{-5} M. Figures of vanadate represent white bars, control (none); hatched bars, 10^{-6} M; black bars, 10^{-5} M.

decrease in cell number. The effect of PD 98059 or dibucaine in decreasing cell number was not induced in transfectants. The inhibitory effect of staurosporine on the proliferation of hepatoma cells was also observed in transfectants.

The presence of wortmannin (10^{-8} to 10^{-6} M), an inhibitor of phosphatidylinositol 3 (PI3)-kinase, caused a significant decrease in hepatoma cells (wild type) cultured for 72 h with 10% FBS (Fig. 4). The inhibitory effect of wortmannin on cell proliferation was not seen in transfectants.

Genistein is an inhibitor of protein tyrosine kinase, and vanadate is an inhibitor of protein tyrosine phosphatase. The proliferation of hepatoma cells (wild type) cultured for 72 h with 10% FBS was significantly inhibited in the presence of genistein (10^{-5} M) or vanadate (10^{-5} M) (Fig. 5). The effect of genistein or vanadate in inhibiting cell proliferation was not induced in transfectants.

The effect of Bay K 8644, an agonist of calcium entry into cells, on proliferation of hepatoma cells (wild type) cultured for 72 h with 10% FBS was significantly inhibited in the presence of Bay K 8644 (10^{-6} M) (Fig. 6). The effect was not observed in transfectants.

Effect of Various Inhibitors of Cell Cycle on Proliferation of Hepatoma Cells Overexpressing Regucalcin

The effect of various inhibitors of cell cycle on the proliferation of hepatoma cells is shown in Figure 7. Cells were cultured for 72 h in a medium containing either vehicle, sodium butyrate (8.3×10^{-5} or 8.3×10^{-4} M), roscovitine (10^{-7} or 10^{-6} M), or sulforapohane (10^{-7} or 10^{-6} M) in the presence of 10% FBS. The proliferation of hepatoma cells (wild type) was significantly inhibited in the presence of butyrate (8.3×10^{-4} M), roscovitine (10^{-7} or 10^{-6} M), or sulforaphane (10^{-7} or 10^{-6} M). The effect of roscovitine (10^{-7} or 10^{-6} M) or sulforaphane (10^{-7} M) in inhibiting proliferation of wild-type cells was not induced in transfectants. The proliferation of transfectants was significantly inhibited in the presence of butyrate (8.3×10^{-4} M) or sulforaphane (10^{-6} M).

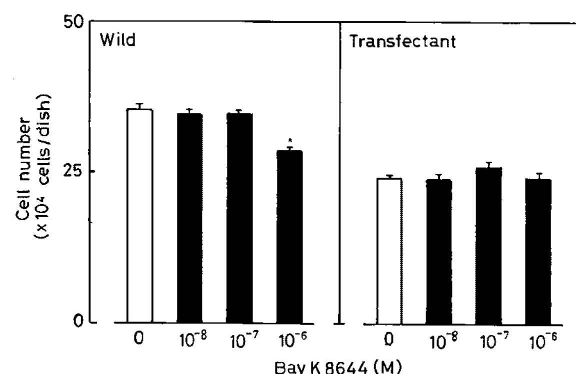

Fig. 6. Effect of Bay K8644 on the proliferation of cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells. Cells (1×10^5) were cultured for 72 h in a medium containing either vehicle or Bay K8644 ($10^{-8} - 10^{-6}$ M) in the presence of 10% FBS, and the number of cells was measured. Each value is the mean ± SEM of six experiments. $^*P < 0.01$ compared with the control (none) value.

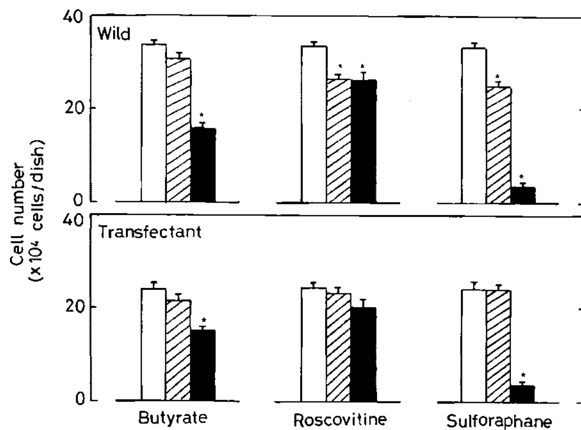

Fig. 7. Effect of butyrate, roscovitine, or sulforaphane on the proliferation of cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells. Cells (1×10^5) were cultured for 72 h in a medium containing either vehicle, butyrate (8.3×10^{-5} or 8.3×10^{-4} M), roscovitine (10^{-7} or 10^{-6} M), or sulforaphane (10^{-7} or 10^{-6} M) in the presence of 10% FBS, and the number of cells was measured. Each value is the mean ± SEM of six experiments. $^*P < 0.01$ compared with the control (none) value. Figures of butyrate represent white bars, control (none); hatched bars, 8.3×10^{-5} M; black bars, 8.3×10^{-4} M. Figures of roscovitine represent white bars, control (none); hatched bars, 10^{-7} M; black bars, 10^{-6} M. Figures of sulforaphane represent white bars, control (none); hatched bars, 10^{-7} M; black bars, 10^{-6} M.

Change in Gene Expression of Cell Cycle-Related Proteins in Hepatoma Cells Overexpressing Regucalcin

The change in mRNA expression of cell cycle-related proteins (cdc2a, chk2, or p21) in hepatoma H4-II-E cells overexpressing regucalcin is shown in Figure 8. Hepatoma cells was cultured for 72 h in a medium containing 10% FBS. The results of RT-PCR analysis using specific primers showed that p21 mRNA levels were significantly increased in transfectants. Meanwhile, the expression of cdc2a, chk2, or G3PDH mRNAs was not significantly changed in transfectants.

Change in IGF-I mRNA Expression in Hepatoma Overexpressing Regucalcin

Hepatoma cells were cultured for 72 h in a medium containing 10% FBS, and the expression of IGF-I mRNA was examined (Fig. 9). IGF-I mRNA was expressed in the hepatoma H4-II-E cells (wild type). This expression was significantly suppressed in transfectants overexpressing regucalcin. G3PDH mRNA levels were not significantly changed in transfectants.

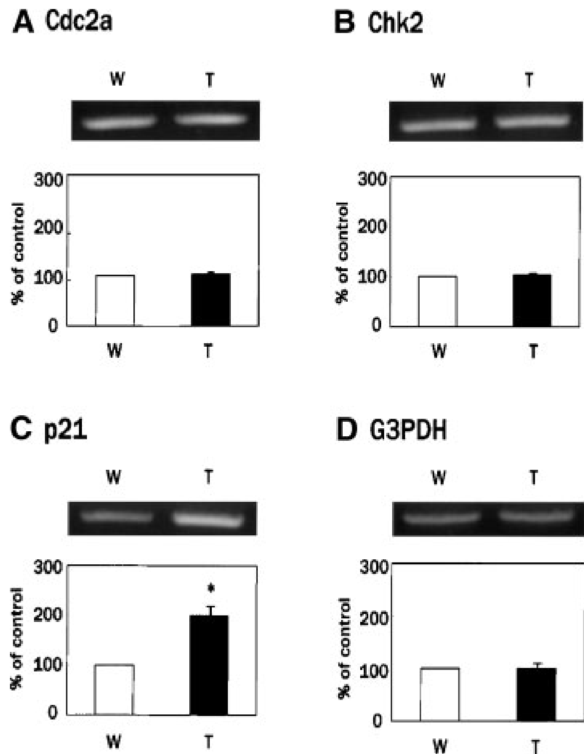

Fig. 8. Change in cdc2a, chk2, or p21 mRNAs expression in the cloned rat hepatoma H4-II-E cells (wild-type; W) or regucalcin/pCXN2- transfected cells (T). Cells (1×10^5) were cultured for 72 h in a medium containing 10% FBS. Total RNAs (2 μg) extracted from the cells were analyzed by RT-PCR using specific primers. The figure shows one of four experiments with separate samples. The densitometric data for cdc2a, chk2, p21, or G3PDH mRNA levels were indicated as % of control (mean ± SEM of four experiments). $^*P < 0.01$ compared with the control value.

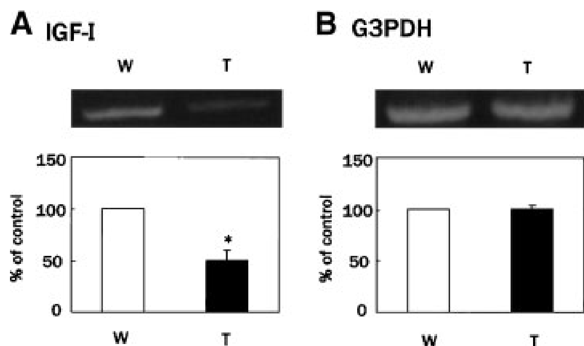

Fig. 9. Change in IGF-I mRNAs expression in the cloned rat hepatoma H4-II-E cells (wild-type; W) or regucalcin/pCXN2-transfected cells (T). Cells (1×10^5) were cultured for 72 h in a medium containing 10% FBS. Total RNAs (6 μg) extracted from the cells were analyzed by RT-PCR using specific primers. The figure shows one of four experiments with separate samples. The densitometric data for IGF-I or G3PDH mRNA levels were indicated as % of control (mean ± SEM of four experiments). $^*P < 0.01$ compared with the control value.

DISCUSSION

Regucalcin has been shown to have a suppressive effect on liver cell proliferation. The expression of regucalcin mRNA is enhanced in the proliferative cells after partial hepatectomy in rats [Yamaguchi and Kanayama, 1995], and the translocation of regucalcin into the nucleus is increased in regenerating rat liver [Tsurusaki and Yamaguchi, 2002b]. Endogenous regucalcin has been shown to prevent the enhancement of nuclear DNA and RNA synthesis in regenerating rat liver, suggesting that the protein regulates proliferation of liver cells [Tsurusaki and Yamaguchi, 2002a,b]. Overexpression of regucalcin has been shown to suppress the proliferation of cloned rat hepatoma H4-II-E cells [Misawa et al., 2002]. Regucalcin is found to bind nuclear proteins or DNA in vitro [Tsurusaki and Yamaguchi, 2003a], and overexpression of regucalcin suppresses the expression of oncogene *c-myc*, Ha-*ras*, or *c-src* and enhances the tumor suppressor genes *p53* and *Rb* mRNA expressions in the cloned hepatoma cells [Tsurusaki and Yamaguchi, 2003b]. Moreover, we found that the suppressive effect of regucalcin on the proliferation of cloned rat hepatoma cells is partly mediated through its regulation for various intracellular signaling-related factors, and that regucalcin enhances p21 mRNA expression and suppresses IGF-I mRNA expressin in the hepatoma cells.

The effect of DRB, an inhibitor of transcriptional activity, or cycloheximide, an inhibitor of protein synthesis, in inhibiting the proliferation of hepatoma cells (wild-type) was not found in hepatoma cells overexpressing regucalcin. Regucalcin has been shown to have a suppressive effect on cytosolic protein synthesis [Yamaguchi and Mori, 1990] and nuclear RNA synthesis [Tsurusaki and Yamaguchi, 2002b] in rat liver. If overexpression of regucalcin inhibits protein and RNA syntheses in transfectants, cycloheximide or DRB with the concentration used in this experiment may not have additional effect. This finding suggests that the effect of regucalcin is partly mediated through the suppression of protein and RNA syntheses in the cells. Other mechanisms by which regucalcin has a suppressive effect on cell proliferation may be found, however.

The proliferation of hepatoma cells (wild-type) was inhibited in the presence of PD98059, dibucaine, staurosporine, or genistein, which is an inhibitor of various protein kinases. The inhibitory effect of PD98059, dibucaine, and genistein on cell proliferation was not revealed in transfectants. Regucalcin has been shown to have an inhibitory effect on various protein kinases in rat liver cytosol and nucleus [Yamaguchi, 2005].

If endogenous regucalcin suppresses the activities of various protein kinases in transfectants, it is possible that the present of various protein kinase inhibitors does not have additional effect on protein kinases. The effect of regucalcin in suppressing cell proliferation may be partly related to its inhibitory effect on MAP kinase, Ca^{2+}/calmodulin-dependent kinase and protein tyrosine kinase in hepatoma cells, and the regucalcin effect may not depend on protein kinase C.

Wortmannin is known to have an inhibitory effect on PI3-kinase. The proliferation of hepatoma cells was inhibited by wortmannin. This effect, however, was not observed in transfectants. It is speculated that regucalcin inhibits PI3-kinase and that it suppresses cell proliferation in hepatoma cells, although it is unknown whether regucalcin has an inhibitory effect on PI3-kinase.

Vanadate is an inhibitor of protein tyrosine phosphatase [Hunter, 1995]. Endogenous regucalcin has been shown to suppress the enhancement of protein tyrosine phosphatase activity in the proliferation of cloned rat hepatoma H4-II-E cells [Inagaki and Tsurusaki, 2000]. The proliferation of hepatoma cells (wild type) was significantly inhibited by vanadate. The effect of vanadate was not seen in transfectants, suggesting that the inhibitory effect of regucalcin on protein tyrosine phosphatase activity is partly involved in the suppression of cell proliferation.

Bay K 8644 is an agonist of calcium entry into cells. The proliferation of hepatoma cells (wild type) was inhibited in the presence of Bay K8644. This effect of Bay K8644 was not seen in transfectants. Regucalcin has a role in the maintenance of intracellular calcium homeostasis in many cell types [Yamaguchi, 2005].

Overexpression of regucalcin was found to suppress the inhibitory effect of various factors, which induce cell-cycle arrest, on the proliferation of hepatoma cells (wild type). The effect of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinase cdc2, cdk2m, and cdk5 [Meijer et al., 1997], or sulforaphane,

which can induce G2/M phase cell cycle arrest [Singh et al., 2004], in inhibiting the proliferation of wild-type cells was not observed in transfectants. Sulforaphane with a higher concentration caused a decrease in cell number of transfectant, suggesting that the chemical induces cell death and apoptosis (data not shown). Meanwhile, butyrate induced an inhibition of the proliferation of wild-type cells and transfectants. Roscovitine can arrest in G1 and accumulate in G2 of cell cycle. Butyrate induces an inhibition of G1 progression. The inhibitory effect of roscovitine or sulforaphane on cell proliferation may not be seen in transfectants, if regucalcin has a suppressive effect on the same pathway which roscovitine or sulforaphane has an inhibitory effect on cell proliferation. Regucalcin has been shown to inhibit nuclear DNA synthesis in regenerating rat liver with proliferative cells [Tsurusaki and Yamaguchi, 2002a]. Presumably, regucalcin induces G1 and G2/M phase cell cycle arrest in hepatoma cells.

The expression of p21 mRNA was found to enhance in transfectant overexpressing regucalcin, although cdc2a and chk2 (checkpoint-kinase 2) mRNA levels were not changed in transfectants. p21 is an inhibitor of cyclin-dependent kinases (cdk). It is speculated that regucalcin enhances p21 expression and that it inhibits G1 progression in hepatoma cells. It cannot exclude the possibility, however, that regucalcin inhibits cdk activity in the cells.

Overexpression of regucalcin suppressed the expression of IGF-I mRNA in the cloned rat hepatoma cells. IGF-I is a growth factor in cell proliferation. It is assumed that regucalcin has a suppressive effect on IGF-I expression in hepatoma cells, and that the suppressed expression leads to the retardation of cell proliferation.

From the previous and present observations, it is assumed that the suppressive effect of regucalcin on cell proliferation is related to its inhibitory effect on the activities of various protein kinases and protein phosphatases, calcium-dependent signaling factors, nuclear DNA, RNA, and protein synthesis or IGF-I expression, and its activatory effect on p21, an inhibitor of cell cycle-related protein kinases. Overexpression of regucalcin has been shown to enhance the expression of p53 mRNA in the cloned rat hepatoma H4-II-E cells [Tsurusaki and Yamaguchi, 2003b]. p53 is known to stimulate p21 mRNA expression to induce cell-cycle arrest. The present study further supports the view that regucalcin plays a role as a regulatory protein in many intracellular signaling pathways in cells. Regucalcin has a suppressive effect on cell proliferation due to hormonal stimulation. Presumably, regucalcin plays a physiologic role in the maintenance of homeostasis of cellular response for cell stimulation.

In conclusion, it has been demonstrated that overexpression of regucalcin suppresses cell proliferation which is mediated through various intracellular-related factors, and that the effect is partly involved in the change in p21 or IGF-I mRNA expression in the cloned rat hepatoma H4-II-E cells. This study further supports the view that regucalcin plays an important role as a suppressor in the enhancement of cell proliferation.

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